[redacted]

Toxicity Study of [redacted]

28-days oral toxicity test in Wistar rats

>> Study Report [redacted] <<

- GLP -

Final version: [redacted]

Sponsor:
[redacted]

Test Facility:
[redacted]

Study Monitor:
[redacted]

Study Director:
[redacted]

All numerical denotations in this study plan follow German conventions.

This study report contains 29 pages without appendices.

[redacted]

SIGNATURES

STUDY DIRECTOR:

[redacted]

[redacted]

TEST FACILITY MANAGER:

[redacted]

[redacted]

DIRECTOR QUALITY MANAGEMENT:

[redacted]

[redacted]

STUDY MONITOR:

[redacted]

[redacted]

All raw data generated by Principal Investigators have been archived with the study raw data. Copies of the signed summary reports were attached to the appendix of this report.

[redacted]

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APPENDIX

ABBREVIATIONS

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BGBl	Bundesgesetzblatt (German federal law gazette)
BW	Body weight
DRF	Dose range finding
ID	Identification (number)
NAD	No abnormality detected
n.e.	Not examined
PI	Principal Investigator
SOP	Standard Operating Procedure
SPF	Specific pathogen free

STATEMENT OF GLP-COMPLIANCE

Toxicity Study of [redacted]

28-days oral toxicity test in Wistar rats

[redacted] **study report** [redacted]

This study was performed according to the following guideline:

- OECD: Guideline for the Testing of Chemicals; Section 4: Health Effects: 407 Repeated Dose 28-day Oral Toxicity Study in Rodents. Updated Guideline, adopted 03th October 2008.

[redacted] is a GLP and GMP certified test facility. A copy of the GLP certificate is attached to this report. The Quality Assurance during this study followed the principles of:

- 'Chemikaliengesetz' (Chemicals Act) of the Federal Republic of Germany (ChemG) §19 and annexes 1 and 2 in the codified version of 02 July 2008 [BGBl. I S. 1146 ff (Nr. 28)], as amended on 02 November 2011 [BGBl. I S. 2162 ff (Nr. 56)], valid from 09 November 2011.
- 'OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring' (as revised in 1997) [ENV/MC/CHEM (98)17], 21 January 1998, and subsequent advisory/consensus OECD GLP documents 2-15 (where appropriate).

For technical reasons, haematology and blood biochemistry data in this study were determined by a Principal Investigator [redacted] not officially certified according to [redacted] maintains a routine quality system to ensure GLP-comparable quality of the test results. Principal Investigator 2 [redacted] is a GLP-certified test site.

There were no deviations from GLP guidelines during the course of this study. Documentation and archiving of raw data and related documents are compliant with current GLP regulations.

[redacted]

[redacted]

QUALITY ASSURANCE STATEMENT

Based on a Quality Assurance review, it was concluded that this report is complete and accurately reflects the data of the study. The results of study parts conducted by the Principal Investigator not officially certified to GLP have been additionally inspected by the quality assurance and were assessed to basically comply with the principles of GLP. The study was performed according to GLP. All audit comments have been satisfactorily resolved.

Toxicity Study of [redacted]

28-days oral toxicity test in Wistar rats

[redacted] **study report** [redacted]

Inspections and reports:

Date of inspection	Criteria	Date of approval/report to the Study Director and the Management
[redacted]	**Study plan final** In-life: animal housing, IRWIN test In-life: necropsy, organ and tissue preservation Histology slide preparation and transfer to Principal Investigator Raw and calculated data, inspection and approval of data and reports provided by Principal Investigators, study report 1st draft study report 2nd draft **Study report final**	[redacted]

Appro[redacted]

SUMMARY

Aim of the study:

The aim of this study was to assess data on the subacute toxicity of the chemical substance [redacted] suspended in water.

Experimental model:

The test item [redacted] was administered daily by oral gavage at dose levels of 111, 333 and 1000 mg/kg body weight per day to 5 male and 5 female Wistar rats per dose group over a period of 28 days. Another 5 male and 5 female rats received the same volume of sterile water as vehicle control. Of these solutions, 5 ml/kg body weight were administered at each application.

Assessed parameters:

During the in-life phase, viability, general and detailed clinical signs, food and water consumption as well as body weight were recorded.

At the end of the in-life phase, grip strength and reactivity to sensory stimuli (beam walking test) were determined and blood samples from all animals were collected to provide data on haematology and serum biochemistry. All animals were sacrificed immediately after bleeding and examined by gross necropsy. Selected organ weights were recorded and tissues and organs were preserved according to the study plan. All samples were processed for histopathological examination. The latter was conducted on samples from the high dose groups and the vehicle groups exclusively.

Assessment of the Results:

Detailed clinical signs, food consumption, motoric activity, and reactivity to sensory stimuli showed no distinct abnormalities.

The mean body weight increase of all experimental groups was within the normal range for rats of this strain and age.

Analyses of haematology and serum biochemistry returned normal values in the vast majority of the monitored parameters. Three minor alterations observed in the serum biochemistry of the male high dose and the female low dose group were assessed to be of no toxicological relevance. Also, the reduced grip strength in female medium dose group was regarded a random effect.

As a tendency, an increased water consumption could be observed in at least the male groups. However, this finding could not be correlated with any toxicological alterations.

The morphological and histological examination of the selected organs did not reveal morphological changes considered to be related to the test item. No morphological differences were noted between the groups. All microscopic findings noted in various organs of animals of the high dose group did not distinguish significantly treated rats from control animals or the differences noted were regarded as random events. All findings are considered to be spontaneous in nature and within the normal background pathology commonly seen in rats of this strain and age.

[redacted]

Parameter	Males			Females		
	Low dose	Medium dose	High dose	Low dose	Medium dose	High dose
Detailed clinical signs	NAD	NAD	NAD	NAD	NAD	NAD
Body weight	NAD	NAD	NAD	NAD	NAD	NAD
Food consumption	NAD	NAD	NAD	NAD	NAD	NAD
Water consumption	Mildly increased ▪	Mildly increased ▪	Mildly increased ▪	NAD	NAD	NAD
Grip strength	NAD	NAD	NAD	NAD	reduced ▪	NAD
Beam walking test	NAD	NAD	NAD	NAD	NAD	NAD
Haematology	NAD	NAD	Alanine transaminase elevated ▪	Globulin increased, Albumin/Globulin reduced ▮	NAD	NAD
Serum biochemistry	NAD	NAD	NAD	NAD	NAD	NAD
Necropsy	NAD	NAD	NAD	NAD	NAD	NAD
Organ weight	NAD	NAD	NAD	NAD	NAD	NAD
Histology	n.e.	n.e.	NAD	n.e.	n.e.	NAD

NAD = No abnormality detected
n.e. = not examined
▮ = Marked findings were assessed to be of no toxicological relevance

Table 1 Summary of the results in comparison to the vehicle group

Conclusion:

A daily oral administration of the test item [redacted] to Wistar rats at a dose level of 111, 333 and 1000 mg/kg body weight over a time period of 28 days resulted in only very mild systemic effects in the male high dose group but did not produce any pathological evidence of a local or systemic toxicity of the test item.

[redacted]

1 Preface

1.1 Identification

Title	**Toxicity study of** [redacted] 28-days Oral Toxicity Test in Wistar Rats
Study number	[redacted]
Sponsor	[redacted]
Test facility	[redacted]
Princ. Investigator 1 (Haematology)	[redacted]
Princ. Investigator 2 (Histopathology)	[redacted]

1.2 Responsibilities

Study Director Deputy Study Director Test Facility Manager Quality Assurance Archivist Principal Investigator 1	[redacted]

[redacted]

Quality Assurance at PI 1
Principal Investigator 2
Quality Assurance at PI 2

[redacted]

1.3 Time Schedule

Study initiation date (Study plan final)
Experimental starting date (Acclimatisation)*
First application (day 1)
Necropsy (day 29)
Experimental completion date (histopathology results)
Study report (draft)
Study completion date (Study report final)

[redacted]

*For organisational reasons, acclimatisation started prior to finalisation of the study plan.

1.4 Test Guideline(s)

The procedures, principles, and objectives of this study report are in accordance to the following guideline:

- OECD: Guideline for the Testing of Chemicals; Section 4: Health Effects: 407 Repeated Dose 28-day Oral Toxicity Study in Rodents. Updated Guideline, adopted 03th October 2008.

1.5 GLP-Compliance

The Quality Assurance during this study followed the principles of:

- Chemikaliengesetz (Chemicals Act) of the Federal Republic of Germany (ChemG) §19 and annexes 1 and 2 in the codified version of 02 July 2008 [BGBl. I S. 1146 ff (Nr. 28)], as amended on 02 November 2011 [BGBl. I S. 2162 ff (Nr. 56)], valid from 09 November 2011.
- 'OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring' (as revised in 1997) [ENV/MC/CHEM (98)17], 21 January 1998, and subsequent advisory/consensus OECD GLP documents 2-15 (where appropriate).

1.6 External Test Site(s) / Principle Investigator(s) (PI)

1.6.1 Haematology and serum biochemistry (PI 1)

The external contract laboratory, [redacted] provided the haemogram, the clinical biochemistry and the blood coagulation data. As details regarding haematological examinations are standardised by SOPs and already summarised in this study plan, a separate phase plan issued by PI 1 was not required.

[redacted]

1.6.2 Histopathological examination (PI 2)

The analysis and interpretation of the sections was conducted by the external GLP test site [redacted] As details regarding histopathological examinations are standardised by SOPs and already summarised in the study plan, a separate phase plan issued by [redacted] was not required.

1.7 Archiving

The study data will be archived according to the principles of:

- 'OECD Series on Principles of Good Laboratory Practice (GLP) and Compliance Monitoring', Advisory Document Number 15: Establishment and Control of Archives that operate in Compliance with the Principles of GLP [ENV/JM/MONO(2007)10], effective 11 June 2007.

All raw data, documents, tissue samples, and other data of relevance retrieved or generated by [redacted] including the study plan, all applicable amendments and the final report were stored in the [redacted] archive for 15 years or however long is legally required. After that time period has elapsed, no data will be discarded without the Sponsor's notification.

Archiving of materials with storage requirements differing from conventional storage conditions may produce additional costs. Upon request of the Sponsor, archiving may be assigned to an adequate external GLP archive. Where retention samples of test items, reference items or other materials were taken, they will be stored for only as long as their substance quality allows for evaluation. The procedures for archiving are described by the internal SOPs [redacted]

Storage throughout the study: The Study Director was responsible for storing data in the proper lab-folder, protected from unauthorized access.

Storage after study completion: The Archivist of [redacted] s responsible for storing data in the central GLP archive [redacted] protected from unauthorized access.

1.8 Amendments and Deviations

1.8.1 Deviations

Some data on serum biochemistry, haematology and blood clotting time could not be gained due to unintended clotting of the blood samples. The following samples were affected:

dose group	animal	missing data
male animals		
high dose	11/791/2	hematology
	11/791/4	hematology
low dose	11/793/2	hematology
female animals		
medium dose	11/796/4	hematology
low dose	11/797/1	Hematology
	11/797/4	hematology

Table 2 Summary of missing blood data

Even though some data of the blood were missing (144), the remaining (1496, 91,22%) available data were assessed to be sufficient to evaluate the influence of the test item on the metabolism and the health of the test animals. Therefore, this deviation was rated uncritical for the results of this study.

1.8.2 Amendments

There were no amendments to the present study.

2 Test System

2.1 Test Animals

Species	*Rattus norvegicus*
Strain	Wistar Han (IGS)
Gender	20 males, 20 females
Source	Charles River Sandhofer Weg 7 97633 Sulzfeld, Germany
Total number of animals	40
Age at acclimatisation	8-9 weeks
Health status	Specific pathogen free (SPF)
Pregnancy status females	Nulliparous, non-pregnant

Rationale for chosen test species:

Wistar rats are commonly used and recommended to assess toxicity. For comparison purposes a large number of publications with sufficient historic data are available.

2.1.1 Animal husbandry

Room	OP1
Hygiene status	SPF
Housing conditions	Clean conventional housing: airing with approx. 10 air changes per hour, room climate 22 ± 3°C, relative humidity at 30-70%, artificial lighting 12 h light/12 h dark
Caging	Groups of up to five animals of the same sex in open macrolon cages type 2000P, TechniPlast (size slightly larger than GV-SOLAS Type IV).
Bedding	Lignocel hygienic animal bedding (J. Rettenmaier & Söhne GmbH + Co. KG, 73494 Rosenberg)
Cage enrichment	Wooden gnawing blocks, size medium 10x2x2 cm, debarked aspen wood, NGM E-022 (ABEDD LAB & VET Service GmbH) autoclaved
Diet	Maintenance diet for rats and mice, No. 1324 TPF (Altromin Spezialfutter GmbH & Co. KG, 32791 Lage), *ad lib.*
Water	Sterilised community tap water, *ad lib.*

2.1.2 Allocation

Upon arrival at the test facility, the animals were visually checked for signs of ill health and abnormalities. After that, the animals were acclimatised to the laboratory conditions for seven (males) or eight (females) days. All animals assigned to this study were housed in the same room throughout the entire study.

2.1.3 Stratification and randomisation

This study was designed as a randomised controlled trial (RCT). Upon arrival at the test facility, rats were weighed individually and grouped into three weight clusters per gender.

Single animals from the central clusters were placed consecutively into prepared cages until the cluster was depleted. Animals from the remaining clusters were caged alternately in the same manner.

Prepared cardboard labels displaying the dose groups were attached blindly onto the cages. The results of this pseudo-randomisation were documented in the according laboratory work sheets.

Rationale for randomisation:

Concerning the avoidance of bias during the distribution of test animals, it is in accordance to current scientific knowledge that a pseudo-randomisation as described here was sufficient to generate the modest amount of unpredictability required for preclinical and clinical studies.

2.1.4 Identification

Each animal in a cage was individualised by a single ear punch. Each cage was labelled with a card displaying the cage number, the study number, information on the animals, and the respective dose group.

2.2 Test item

The Sponsor supplied all data on the test item. Chemical analysis of the identity and the purity of the batch was not part of this study. The Sponsor ensured that all test items delivered to the test facility were appropriately tested for identity, strength, purity, stability and uniformity, according to guideline 21 CFR 58.31(d) where applicable.

Identification
Containing (composition)

Supplier
Data sheet
Molecular formula
Molecular weight
Purity
CAS No.

[redacted]

[redacted]

vivo Science ID	[redacted]
Batch No.	
Batch production	
Appearance	
Storage conditions	
Expiry date	
Solubility in water	
Stability	
Hazard information	
Retention sample	
Retention period	

2.2.1 Test item preparation

Identification	[redacted] suspended in sterilized tap water
Storage conditions	Prepared fresh daily
Stability	Unknown
Safety precautions	Suitable protective clothing was worn, dust formation, skin and eye contact was avoided

Instructions for test item preparation:

1. Determine gross vial mass of a screw top vial on an analytical balance
2. Add test item into screw top vial
3. Determine net mass of employed test item
4. Add required volume of water in order to produce the required application dose as stated in the laboratory work sheets
5. Vortex immediately before each dose will be drawn into the application syringe

The test item preparation was intended for an application volume of 5 ml per kg body weight. All material data sheets were stored with the raw data.

2.3 Vehicle

Identification	Sterilised community tap water
Supplier	Stadtwerke Gronau
Batch number	Not applicable
Purity	See appendix D
Storage conditions	Not applicable
Expiry date	Not applicable
Retention sample	None

3 Materials and Methods

3.1 General Study Design

The test item was administered daily at 5 ml/kg BW by oral gavage. Dose levels of either 111, 333 and 1000 mg/kg BW/day were applied to 5 male and 5 female Wistar rats each for 28 days.

Test substances were administered to rats of 8-9 week age at the start of the in-life phase. The first day of application was indicated as day 1.

During the in-life phase, viability, general and detailed clinical signs, body weight, food and water consumption, grip strength and reactivity to sensory stimuli was monitored as scheduled in chapter 3.2.

At the end of the treatment period, blood samples from all animals were drawn to provide data on haematology and serum biochemistry. Subsequently, all animals were sacrificed and examined by gross necropsy. As specified in table 7, tissues and organs were preserved and processed for histopathological examination. Accordingly, organ weights were recorded.

Group no.	Dose group	Number of animals (male/female)	Dose / BW [mg/kg]	Application volume / BW [ml/kg]
1	vehicle	5 / 5	0	5
2	low	5 / 5	111	5
3	medium	5 / 5	333	5
4	high	5 / 5	1000	5

Table 3 Dose groups

Rationale for the dose levels:

In a previously performed dose range finding study[1] with dose escalation, the test item was administered in doses up to 1000 mg/kg body weight over a time period of 16 days and produced no observable toxic effects in the test animals. In accordance with the Sponsor, 1000 mg/kg was determined as high dose for this study.

3.2 Monitoring

The following parameters were observed and documented during the in-life period:

- Viability/mortality — Twice daily
- General clinical signs/behaviour — Daily
- Detailed clinical signs (IRWIN-Test) — Once weekly (including once before beginning of application)
- Grip strength, Beam walking test — Once during the last exposure week
- Body weight — Once weekly (including once before application start)

[redacted]

- Group food consumption	Once weekly (including once before application start)
- Group water consumption	Twice weekly (including once before application start)

3.2.1 Viability, general clinical signs and behaviour

Cage-side observations to detect signs of illness or reactions to treatment, moribund animals or fatalities were conducted as scheduled in chapter 3.2 up to the day of necropsy. In case of any findings, the individual animal would have been examined using a „health status" form sheet according to [redacted]

3.2.2 Fatalities

Moribund animals according to the OECD Guideline "Guidance Document on the Recognition, Assessment, and use of Clinical Signs as Humane Endpoints for Experimental Animals used in Safety Evaluation, ENV/JM/MONO(2000)7" would have been removed when noticed and euthanased humanely after notification of the Study Director or the Deputy. On workdays, recently deceased animals would have been dissected for gross necropsy immediately after detection. On non-workdays, animals found dead would have been stored in a freezer at < -15°C and dissected on the next working day.

After visual examination, the Study Director decided on further test, e.g. measuring organ weights, preservation of additional tissues, and further histopathology.

3.2.3 Detailed clinical signs (IRWIN-test)

Monitoring of individual detailed clinical signs was performed employing a modified IRWIN[2] test on a time schedule described in chapter 3.2 and as specified in detail [redacted] „Clinical observations and functional observation assessment (FOB) on rodents"). Findings were noted on prepared checklists, documenting 30 endpoints in following categories:

- Appearence (general status, physiology, autonomic functions, neurology, tonus, E1-E4)
- Motoric / exploration behaviour (M1-M2)
- Excitation (R)
- Abnormal behaviour (A1-A4)

The test was performed in a standard arena (42 x 26 x14 cm) outside the cage. Endpoints were evaluated with a scoring system ranging from 0 to 5 for each endpoint. Selected endpoints were summarised in diagnostic clusters as displayed in Table 4. For each cluster standard values were defined to discriminate between normal and altered clinical signs. An animal´s condition was considered as altered if the resulting scoring value was outside the (rounded) 80% -120% range of the standard value.

[2] Irwin, S. (1968). Comprehensive observational assessment: Ia. A systematic, quantitative procedure for assessing the behavioral and physiological state of the mouse. Psychopharmacologia (Berl.), 13, 222-257.

		Clinical finding		
	Appearance	ALTERED	NORMAL	ALTERED
E1	General health status	<6: „reduced"	6-10: „NAD"	>10:„reduced"
E2	Physiology / autonomic functions	<8:"conspicuous"	8-12: „NAD"	>12:"conspicuous"
E3	Neurology (Reflexes, ear funktion)	<9: „reduced"	9-15: „NAD"	>15: „increased"
E4	Tonus	<3: „reduced"	3-5: „NAD"	>5: „increased"
	Motor activity, Exploration	ALTERED	NORMAL	ALTERED
M1	Motor activity	<4: „reduced"	4-8: „NAD"	>8: „increased"
M2	Exploratory behavior	<3: „reduced"	3-5: „NAD"	>5: „increased"
	Excitation	ALTERED	NORMAL	ALTERED
R	Excitation	<6: „reduced"	6-10: „NAD"	>10: „increased"
	Abnormal Behaviour	ALTERED	NORMAL	ALTERED
A1	Freezing, Tremor	describtion all findings in words	no scoring applied	describtion all findings in words
A2	Stereotypies, abnormal movement patterns			
A3	Self-mutilation			
A4	Urine and faeces			

Table 4 Evaluation criteria summarised in diagnostic clusters for the IRWIN-test

3.2.4 Grip strength and beam walking test

In the last exposure week, additional recordings were made of the grip strength and reactivity to stimuli (beam walking test[3]).

For the assessment of grip strength, untrained rats gripping a small grid attached to a grip strength meter (TSE 303500, [redacted] were manually pulled away from the grid. The maximum read-out (in grams) was denoted as the individual rat's grip strength.

For the beam walking test, animals were placed on a small wooden bar (5 cm x 130 cm) near a bright light source (electric bulb, 60 W). The placing and the if so faults of the foot placement on the bar were rated in a 7-point scoring system as depicted in table 5.

[3] Goldstein, L.B. and Davis, J.L. (1990). Influence of Lesion Size and Location on Amphetamine-Facilitated Recovery of Beam-Walking in Rats. Behav Neurosci. 104(2):320-7

Observations / findings	Score
the animal refuses to cross the bar or needs more than 90 seconds	0
the animal was not able to place all limbs on the bar	1
the animal placed all limbs on the bar and kept the balance for at least 5 seconds	2
the animal crossed the bar with limbs dragged behind (footslips)	3
the animal crossed the bar (dragged limbs) and placed at least the limb(s) once on the bar.	4
the animal crossed the bar, more than half of the steps are footslips	5
the animal crossed the bar, less than the half and more than two of the steps are footslips	6
the animal crossed the bar with not more than two footslips	7

Table 5 Beam walking scores

A score of 6 or 7 points was regarded as normal. In some instances, it is natural behaviour even for untreated, healthy rats to refuse a crossing of the bar at all, resulting in a scoring of 0 points. In these cases the Study director decided on the final classification of this finding.

These procedures followed [redacted]

3.2.5 Body weight, food and water consumption

Individual body weight, group food and group water consumption were monitored as scheduled in chapter 3.2.

3.3 Terminal procedures

3.3.1 Blood sampling, haematology, serum biochemistry and blood clotting time

On day 29, approx. 1100 µl blood were taken from the retroorbital vein plexus of each animal immediately before sacrificing the animal. In order to avoid coagulation, 450 µl of each blood sample were mixed with 50 µl 10x HEPES-EDTA solution directly after bleeding. 600 µl of the remaining blood were split into two heparine syringes, one of them for serum preparation at [redacted] These sera were retained in a freezer at -20 +/- 5 °C to allow a potential determination of thyroid hormones.

The EDTA blood and the second part of heparine blood samples were transferred to PI 1 to analyse parameters on haematology and serum biochemistry (see table 6) as well as blood clotting time. The samples were individually barcode-labelled at the time of data exchange according to SOP [redacted] (shipment of test items, blood samples, serum samples and materials, internal and external) of [redacted] A courier service transferred the samples directly to PI 1 on the day of blood collection. Blood analysis was performed 8 h after bleeding at maximum. Any surplus blood after analysis was disposed of at the laboratory of PI 1.

[redacted]

The original results of the analysis were returned to the Study Director at [redacted] Data were exchanged on standardised printout-sheets, archived at the test facility with the raw data.

Parameter	Unit
Leukocytes	x 10^3/µl
Erythrocytes	/pl
Reticulocytes	‰
Haemoglobin concentration (HB)	g/dl
Haematocrit (HKT)	%
Mean corpuscular volume (MCV)	fl
Mean corpuscular haemoglobin (MCH)	pg
Mean corpuscular haemoglobin conc. (MCHC)	g/dl
Platelets/thrombocytes	x 10^3/µl
Lymphocytes	%
Monocytes	%
Band neutrophil	%
Segmented neutrophil	%
Eosinophils	%
Basophils	%

Parameter	Unit
Sodium	mmol/l
Potassium	mmol/l
Chloride	mmol/l
Calcium	mmol/l
Glucose (in serum)	mg/dl
Total cholesterol	mg/dl
Urea	mg/dl
Creatinine	mg/dl
Total protein	g/dL
Albumin (in serum)	g/dL
Globulin	g/dL
A/G Ratio	n.a.
Alanine aminotransferase (ALT)[1]	U/l
Aspartate aminotransferase (AST)[2]	U/l
Alkaline phosphatase	U/l
Gamma glutamyl transferase (GGT)	U/l

[1]: designated in the raw data with its alternate name Glutamic-pyruvic transaminase (GPT)
[2]: designated in the raw data with its alternate name Glutamic-oxaloacetic transaminase (GOT)

Table 6 Haematological and serum biochemistry examinations

3.3.2 Gross necropsy, tissue preservation and organ weight

The in-life phase was terminated on day 29. Animals were sacrificed by asphyxiation in CO_2 atmosphere. For each individual animal, a gross necropsy was performed and all occurring lesions were noted. The weights of the tissues marked with "W" in table 7 were taken and the tissues marked with a "P" were preserved in 4% neutral buffered formalin. Data were recorded on prepared postmortem checklists according to SOP [redacted]

[redacted]

[redacted]

Ref. No.	Tissue/organ	Procedure[1]
1	Gross lesions	P
2	Oesophagus	P
3	Trachea and thyroid	P
4	Stomach	P
5	Thymus	P, W
6	Liver	P, W
7	Spleen	P, W
8	Duodenum	P
9	Jejunum	P
10	Ileum (with Peyer's patches)	P
11	Cecum	P
12	Colon	P
13	Rectum	P
14	Lymph nodes (mesenterial)	P
15	Kidney	P, W
16	Vagina	P
17	Adrenals	P, W
18	Urinary bladder	P
19	Testes/ovary	P, W
20	Epididymides	P, W
21	Prostata/uterus/cervix	P (W)
22	Heart	P, W
23	Lungs	P
24	Peripheral nerve	P
25	Bone marrow[2]	P
26	Sternum	P
27	Spinal cord	P
28	Whole brain	W
29	Cerebrum	P
30	Cerebellum	P
31	Pons	P
32	Eye	P
33	Skeletal muscle	P

[1]: P = preservation, W = weight determination
[2]: Bone marrow will only be examined if pathologic changes are observed in the sternum

Table 7 Necropsy, tissue preservation, and organ weights

The organ weight denoted by „W" in table 7 was recorded; tissues denoted by „P" were preserved in the appropriate fixatives.

3.4 Histology

3.4.1 Preparation of histological slides

Histological preparation of tissues denoted by „P" in table 7 was accomplished for animals of the high-dose and the vehicle group.

The selected tissues were embedded in paraffin wax, sectioned, and stained with hemalum and eosin [redacted] histology lab performed the preparation and fixation of the raw materials for histological sectioning according to SOP [redacted]

After that, the stained sections were sent to the Principal Investigator 2 for histopathological examination. Data documentation and transfer were conform to [redacted]

[redacted]

3.4.2 Histopathological Examination

Principal Investigator 2 conducted the analysis and interpretation of the stained sections.

The Pathologist documented all findings during histopathological examination in an expert report. Animal heading data and necropsy findings were taken from the postmortem records documented by [redacted] istopathological observations were entered manually into a computerized system using the software PathData version 6.2 (PathData is a registered trademark of Pathology Data Systems, Inc.). Data were recorded for each individual animal in full descriptive terms. In addition, the incidence of histopathological findings was presented in tabular form. Wherever possible, histological alterations were described according to their distribution, severity, morphological characteristics and severity scores.

The pathology expert report and the original histological slides were returned to the Study Director. The principal section of the expert report was attached to the appendix of this report. Histological slides will be archived for 15 years.

3.5 Statistical Analysis

Spread sheet calculations were performed using Microsoft® Excel® 2011 for Mac.

The body weight was documented for each individual animal. Food- and water consumption were documented sorted by experimental groups. For each experimental group, means and standard deviations were calculated. The test item groups were analysed in comparison to the vehicle group by calculation of statistical significance using a two-tailed, unpaired student´s t-test.

For all calculations, the significance level was set to 0,05.

3.5.1 Descriptive statistics

The arithmetic mean, standard deviation and median were calculated for all grouped numerical data originating from the monitoring of grip strength, gross pathology (organ weights) and parts of the haemogram (for details see appendix). Where appropriate, detailed column statistics were applied (minimum / maximum data, 25% quantiles, std error, upper and lower confidence interval 95%).

Data of the IRWIN- and the beam walking test were analysed using a scoring system as described in chapter 3.2.3 and 3.2.4.

3.5.2 Deductive statistics

The main decision tree documented within the appendix A displays the general approach of deductive statistical analysis. It is accepted scientific knowledge that the numerical (interval-

and ordinal-scaled) biological data collected in the present study are distributed normally (Gaussian).

Most statistical hypotheses in this study are characterised best as "many to one"– a vehicle control vs. three treatment groups. Therefore, the adequate analysis method is a One-Way ANOVA (Analysis of variance), followed by a post hoc t-test.

In case of interval-scaled data, the One-Way ANOVA was supplemented by Dunnett's post-hoc t-test.

The entire deductive statistics were performed using Graph Pad Prism for Mac, Version 5.01c.

All statistical data were documented within the appendix.

4 Results and Discussion

Raw data and summarised data of the results were attached in tabular form in appendix A.

4.1 Monitoring

4.1.1 Viability, general clinical signs and behaviour

In the female low dose group two animals showed a minor injury on their back. For animal 11/797/0 this was observed from [redacted] and for animal 11/97/1 on [redacted] Afterwards, these injuries were not detectable any more. Injuries like the detected are implications of natural hierarchic encounters. This was not regarded to be a treatment related effect. No further abnormalities concerning general clinical signs or behaviour were observed.

4.1.2 Fatalities

All animals survived until the planned end of the study.

4.1.3 Detailed clinical signs (IRWIN test)

In the course of this study, no signs of illness (e.g. changes in skin, secretions), autonomic activity (e.g. lacrimation, piloerection), stereotypies or behavioural reactions (e.g. self-mutilation) in response to the treatment or otherwise were observed. Only one animal of the male low dose group (11/793/2) showed a slightly reduced excitation in the third week of treatment. The following week this incident was not detectable any more. Animals of the higher dose groups showed no abnormalities at all. This incident was regarded as coincidental, a treatment related effect could be excluded.

4.1.4 Grip strength and beam walking test

Motoric activity and reactivity to visual and proprioceptive stimuli were not altered by the administration of the test item (no score less than 6). No animal refused to cross the bar. A test item related effect could not be detected.

The grip strength of male and female animals from all treated groups and the vehicle control group showed only one significant difference. The animals of the female medium dose group showed a lower grip strength than the vehicle group. No other treatment group showed any alteration concerning the grip strength. Due to this, this incident was regarded to be a random effect.

4.1.5 Body weight

The mean body weight gain of male and female animals from all treated groups and the vehicle control group was within normal range for rats of this strain, gender and age. Only the **relative** bodyweight (percent of the initial body weight) of the female high dosage group at day 29 was significant lower than the bodyweight of the vehicle animals at the same time

point. This effect was not detectable in any other group or at any other time point. Due to this, this incident was regarded as a random effect with no correlation to the treatment.

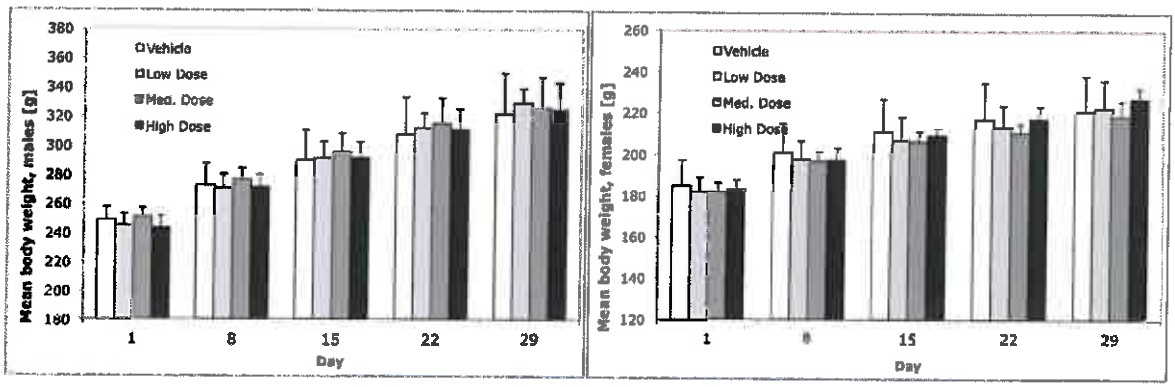

Figure 1 **Mean absolute body weight [g]** of the male (left) and the female (right) animals, sorted by groups and treatment day (n=7). Means and standard deviations of each group are given.

4.1.6 Food consumption

The mean food consumption of male and female animals from all treated groups and the vehicle control group was within normal range for rats of this strain and age.

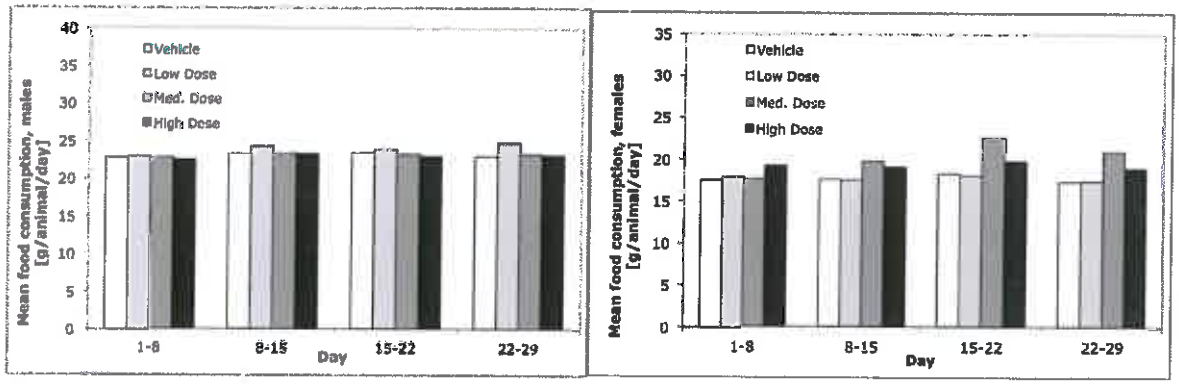

Figure 2 **Mean food consumption [g]** of the male (left) and the female (right) animals, per animal and day, sorted by experimental groups and treatment period (7 days).

4.1.7 Water consumption

The mean water consumption of all animals of all experimental groups was within normal range for rats of this strain and age, albeit a mildly dose dependent increase in water consumption was detectable at least in the male animals.

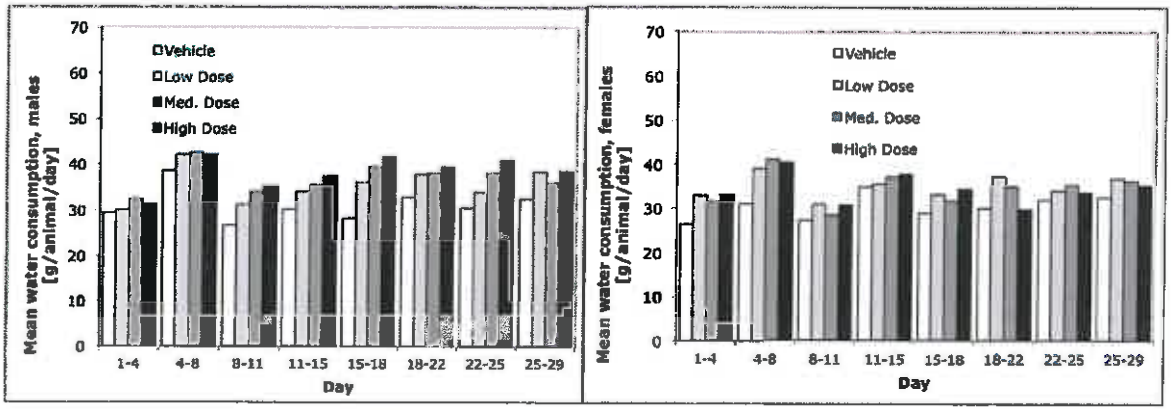

Figure 3 **Mean water consumption [g]** of the male (left) and the female (right) animals, per animal and day, sorted by experimental groups and treatment period (7 days).

4.2 Haematology and serum biochemistry

Analysis of haematology and serum biochemistry showed normal results in the vast majority of parameters monitored.

The ALT (alanine transaminase) activity of the male high dose animals was statistically significant increased. No other treatment group was affected. Neither the livers nor the hearts of this groups showed any histopathologic alteration or a change of organ weight so this statistical increase was not induced by a necrosis of tissue cells. In addition, the ALT values of the male high dose animals were within the normal range of healthy animals of this age (historical data, 20-42 U/l). Therefore, this alteration was regarded not to be treatment related.

The globulin amount of the female low dose animals was increased. As a consequence, the respective albumin-globulin ratio decreased. However, there was no indication of inflammation in the liver or elsewhere during necropsy or after histopathologic examination. Due to this, a treatment related effect could not be detected.

All other biochemical parameters and the blood cell counts showed no significant differences among the groups.

4.3 Necropsy

At macroscopic examination, no relevant pathological findings were noted for the test animals. The organ weight analysis showed normal results for most of the organs investigated.

Only few alterations were observed:

- The right adrenals of the male rats of the low dose group animals showed a significantly lower weight in comparison to the vehicle group.
- The thymus tissues of the medium male group were of significant lower weight.
- The axillar lymphnodes of the female dosage groups were of significant higher weight as the lymphnodes of the vehicle group (high dose and medium dose significance $p<0,05$, low dose $p<0,01$)
- Some animals of the female vehicle group showed findings during the necropsy. Animal 11/794/1 showed a reddened lymphnode near the left kidney, animal 11/794/2

a reddened thymus and animal 11/794/4 some red dots at the thymus.

Only the right adrenals of the male low dose group showed a significant lower weight, the left adrenal weights of the same group were not reduced, nor showed any other treatment group any signs of adrenal weight reduction or other alterations. An indication for treatment caused alteration was not detectable neither during necropsy nor by histopathologic examination. Due to this a test item relation could be excluded.

The thymus weights of the male medium dose animals were significantly lower in comparison to the vehicle group of this study (group 11/794). In comparison historical data no significant reduction of thymus weight was detected. There were no hints of alteration of the thymus tissues during the necropsy, but two of the animals of the vehicle group (11/794/2 and 11/794/4) showed a reddened or dotted thymus. Except for data of this two animals, no significant reduction of weight was calculated any more. A test item relation was not detectable.

The axillar lymphnodes of the female vehicle control animals were observed to be at unusual low weights. This was regarded as an accumulated random effect (statistical α-error, probably due to the small sample size). Therefore, statistical significant differences returned by calculations for the respective treatment groups were considered to be of no biological relevance. The weights determined for the axillar lymphnodes of all animals from the female treatment groups were within normal range for rats of this strain and age.

No other relevant changes were observed during necropsy.

4.4 Histology

At histopathologic examination, a small number of microscopic findings were recorded in the examined organs. These findings were described in detail in the Pathology Report (for details see appendix B).

As a result of the assessment given in the histopathology expert report, the type, incidence and severity of all microscopic findings noted did not indicate a relationship to the treatment with the test item. All alterations were regarded to be spontaneous in nature and within the normal background pathology commonly seen in rats of this strain and age.

4.5 Summary of results

Table 8 summarises the results of the treatment groups compared to their vehicle control groups.

Parameter	Males			Females		
	Low dose	Medium dose	High dose	Low dose	Medium dose	High dose
Detailed clinical signs	NAD	NAD	NAD	NAD	NAD	NAD
Body weight	NAD	NAD	NAD	NAD	NAD	NAD
Food consumption	NAD	NAD	NAD	NAD	NAD	NAD
Water consumption	Mildly increased ▪	Mildly increased ▪	Mildly increased ▪	NAD	NAD	NAD
Grip strength	NAD	NAD	NAD	NAD	reduced ▪	NAD
Beam walking test	NAD	NAD	NAD	NAD	NAD	NAD
Haematology	NAD	NAD	Alanine transaminase elevated ▪	Globulin increased, Albumin/Globulin reduced ▮	NAD	NAD
Serum biochemistry	NAD	NAD	NAD	NAD	NAD	NAD
Necropsy	NAD	NAD	NAD	NAD	NAD	NAD
Organ weight	NAD	NAD	NAD	NAD	NAD	NAD
Histology	n.e.	n.e.	NAD	n.e.	n.e.	NAD

NAD = No abnormality detected
n.e. = not examined
▮ = Marked findings were assessed to be of no toxicological relevance

Table 8 Summary of the results in comparison to the vehicle group

5 Assessment and conclusion

Detailed clinical signs, food consumption, motoric activity, and reactivity to sensory stimuli showed no distinct abnormalities.

The mean body weight increase of all experimental groups was within the normal range for rats of this strain and age.

Analyses of haematology and serum biochemistry returned normal values in the vast majority of the monitored parameters. Three minor alterations observed in the serum biochemistry of the male high dose and the female low dose group were assessed to be of no toxicological relevance. Also, the reduced grip strength in female medium dose group was regarded a random effect.

As a tendency, an increased water consumption could be observed in at least the male groups. However, this finding could not be correlated with any toxicological alterations.

The morphological and histological examination of the selected organs did not reveal morphological changes considered to be related to the test item. No morphological

[redacted]

differences were noted between the groups. All microscopic findings noted in various organs of animals of the high dose group did not distinguish significantly treated rats from control animals or the differences noted were regarded as random events. All findings are considered to be spontaneous in nature and within the normal background pathology commonly seen in rats of this strain and age.

A daily oral administration of the test item, [redacted] to Wistar rats at a dose level of 111, 333 and 1000 mg/kg body weight over a time period of 28 days resulted in only very mild systemic effects in the male high dose group but did not produce any pathological evidence of a local or systemic toxicity of the test item.

6 Distribution

This report will be distributed as follows:

[redacted]	Original
Study Monitor:	1 authorised copy

[redacted]

[redacted]